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13. ABSTRACT *(Maximum 200 words)*

The objective of this research was to test promising compounds for antileishmanial activity and to test for potential host toxicity with use of human CEM T4 cells. Growth experiments and testing for inhibition of parasite unique enzyme systems were employed in this study.

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MILITARY SIGNIFICANCE

The need for leishmanicides cannot be overemphasized. At present chemotherapy is dependent on a relatively small number of synthetic drugs. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to others. In the chemotherapy of visceral and cutaneous leishmaniasis, the choice of drugs is very limited and success of a particular drug appears to vary from locality to locality, presumably due to strain differences in Leishmania.

To date the logical design of antiparasitic drugs has proved largely unsuccessful with the exception of purine metabolism in protozoa. While mammalian cells are capable of de novo synthesis of purines, many parasites do not synthesize purines but use salvage pathways. Analogues inhibiting key enzymes in purine pathway should, therefore, provide novel therapeutic agents. Purines and pyrimidines serve not only as precursors of RNA and DNA, but also as stores of high energy phosphate, constituents of certain coenzymes, and modulators of various enzymatic reactions. In view of this vital role, intervention of their metabolism will have profound effects on the organism.

To date there is no safe, effective, and quality-controlled antiparasitic vaccines. Membrane antigens differ from one species to another and during the course of infection, making the production of a useful vaccine very difficult.

The elucidation of the biochemical mode of action of promising compounds and the identification of unique enzyme systems will permit the logical design of more effective derivatives and also will provide insight on the mechanism of drug resistance. This information may allow a therapy program to be developed which would decrease or eliminate the problem of drug resistance.

Targeting of already promising compounds may increase the efficacy of these compounds for the various disease states of leishmaniasis and be more cost effective than the development of more than one drug.

Targeting will also allow the reduction in toxicity of certain compounds, and also be more cost effective since less drug should be required.

INTRODUCTION

Leishmaniasis is caused by protozoan parasites of the Order Kinetoplastida: Family-Trypanosomatidae. The disease is estimated to affect 12 million people in Third World countries. Leishmania extracellular forms (promastigotes) are injected into human skin during bites by the sandfly vector. Promastigotes are phagocytized by reticuloendothelial cells, within which the parasites transform into intracellular amastigotes. Human disease results from multiplication of amastigotes within macrophages. Present therapy with pentavalent antimony is potentially toxic, and often ineffective. One rationale for searching for alternative treatment is to identify a unique enzyme system and to target this system for chemotherapeutic exploitation.

Many of the enzymes involved in the synthesis of nucleic acids of the parasitic protozoans have been found to be unique, and for this reason we have begun studies to compare the DNA synthetic enzymes of parasitic protozoa to the mammalian polymerases.

During this research year we rested a number of compounds for Walter Reed Institute of Research (WRAIR) for antileishmanial activity.

We have developed a human CEM T_4 in vitro assay to determine the toxicity of promising antileishmanial compounds for host cells. Because T_4 cells are extremely important in eliciting the immune response, it is of profound importance that these cells are not compromised during chemotherapy of a parasitic disease. Use of our assay system can save WRAIR expensive and time-consuming *in*

vivo animal testing and provide critical data on promising compounds.

We also continued our investigations of the DNA polymerases of the leishmanial parasite because (1) these enzymes are unique from host enzymes and (2) they are extremely important in parasite survival. Compounds which are shown to be inhibitory to leishmanial DNA polymerases and exhibit low toxicity in the CEM T_4 system are potential candidates as therapeutic agents.

METHODS

Cultures of parasitic protozoa. Promastigotes of *Leishmania mexicana* Walter Reed strain 227, were maintained in this laboratory in tissue culture flasks containing the defined medium of Steiger and Black (1) supplemented with 5% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, New York) and 50 mg/L gentamycin. The cells were grown at 26°C and subcultured weekly.

Cultures of T_4 cells. Human lymphocyte CEM T_4 cells were obtained from the Department of Pharmacology at the University of Massachusetts Medical Center. They were cultured in tissue flasks containing RPMI 1640 medium (Sigma Chemical Co., St. Louis, Missouri) supplemented with 0.1% sodium Bicarbonate, 5% heat-inactivated fetal calf serum, and 50 mg/L gentamycin. Cultures were incubated in a 5% CO_2 chamber at 36C subcultured semi-weekly.

Assay inoculum. *Leishmania sp.* and T_4 cells were diluted with fresh medium 24 h prior to use to ensure a log phase culture. The inoculum was standardized at the start of the assay with a spectrophotometer (Spectronic 21, Bausch & Lomb, Rochester, New York) in order to eliminate variations caused by different concentrations of cells growing at varying rates, and thus being inhibited differentially owing to cell concentration. Cell stock was centrifuged in a microcentrifuge at 200 - 400 *g* for 3 minutes and resuspended in fresh medium to give an initial cell concentration of approximately 5 X 10^5/ml in the test wells.

Model for microwell plate assay procedure. Assays were performed in Corning sterile covered polystyrene 96-well round bottom tissue culture plates that were not tissue culture treated.

This is very important because cells will adhere to the surfaces of treated wells, and the absorbance readings will be inaccurate. All wells contained a uniform total volume for the assay.

Blank wells contained equal volumes of medium and sterile deionized double-distilled water. Control wells received medium, water, and inoculum, while test wells received the increasing amounts of test compound replacing water. Six replicates of each level of test compound were made. The standardized inoculum was stirred gently, under aseptic conditions, in a deep Petri dish, and suitable aliquots were pipetted into all but the blank plate wells. Absorbance readings were taken on a microplate spectrophotometer (Microplate Reader, Model MR 600, Dynatech Laboratories, Inc., Alexandria, Virginia) set in single wavelength mode with a suitable filter (490, 660 nm). The plate was shaken on a Vortex Genie-2 fitted with a 6-inch (15-cm) platform head containing a 96-well plate insert in order to ensure suspension of the cells just prior to reading the absorbance. Microwell plates containing leishmania were incubated in a 26°C incubator and read at 0, 24, 48, and 72 h. Microwell plates containing T_4 cells were incubated at 37°C with 5% CO_2 for the desired time. Toxicity studies were usually monitored at 0 and 72 h. Cellular toxicity was measured by determining the IC_{50} (that concentration of an agent causing 50% inhibition compared with controls.)

To determine whether trubidity, observed photometrically in the microwells, would have a direct relationship to the cell concentration, we added several dilutions of cultures of Leishmania cells and human CEM T_4 cells to microwells. Absorbance was

measured with a microplate reader, and the well contents were counted on the Coulter counter, which had been calibrated against a hemocytometer for each cell line.

As a further check of the accuracy of this rapid method, we compared the IC_{50} of pentamidine, a known antileishmanial agent [2], by the microwell methods and the test tube method.

Test tube assay procedure. The assay procedure, a modification of the method of Kidder and Dewey [3], has been used for drug screening regularly in this laboratory. Scratch-free pyrex screw cap tubes (16 X 150 mm) were selected to match as closely as possible for use in the assay. The medium of Steiger and Black, supplemented with test compounds or water in a total volume of 5 ml, was used. The tubes (in triplicate) were incubated with loose caps in a slanted position (5°) in an incubator at 26°C for 72 h. The tubes were vortexed before reading the absorbance at 660 nm, by use of a spectrophotometer equipped with a test tube chamber.

Cell counts using a Coulter counter. Aliquots from wells were counted at time 0 and 72 h with Model ZF Coulter counter (Coulter Electronics, Hialeah, Florida) with settings of 1/amp = 0.707 and 1/aperture current = 2 for the protozoan assays and 0.707, 16, respectively, for the T_4 lymphocyte assays.

Cell culture conditions for enzyme isolations. Promastigotes of Walter Reed strain 227 were used in these experiments. This strain has been previously identified as *Leishmania mexicana*

amazonensis (J. Decker-Jackson and P. Jackson, personal communication) and was obtained from the Leishmania Section of the Walter Reed Army Institute of Research. Promastigotes, were grown in brain heart infusion medium containing 37 g of brain heart infusion medium (Difco Laboratories, Detroit, Mich.) liter of water^{-1}, 10% heat inactivated serum, and 26 μg of hemin ml^{-1}. Cells were grown at 26°C in 2,000-ml wide Fernbach flasks containing 250 ml of brain heart infusion medium. Cells were harvested after 4 days during the exponential growth phase. The cell density was 4 X 10^7 to 6 X 10^7 cells ml^{-1}.

Protein assays. Protein concentrations were determined by either the dye-binding method (Bio-Rad laboratories, Richmond, Calif.) or a modified method. The modified method was performed in 96-well microplates by adding 80 ml of Bio-Rad dye and 20 μl of a column fraction. The plate was them read in a Dynatech 600 miroplate reader at 575 nm.

Isolation and assay of S-Adenosylmethionine Synthetase. Using the method of Hoffman and Kunz (4), we optimized our enzyme assay for *L. mexicana* 227 promastigotes. Methionine adenosyltransferase activity was measured at 35°C in 100 μl of a standard assay mixture containing 150 mM KCl, 20 mM $MgSO_4$, 5 mM dithiothreitol, 50 mM Tris (pH 7.5), 5 mM ATP, and 10 μM L-[^{14}C]methionine. The cationic [^{14}C]adenosylmethionine formed was isolated by spotting 80-μl portions of reaction mixtures on 2.3-cm-diameter disks of Whatman P81 cellulose phosphate cation-exchange paper, removing unreacted methionine by washing in a beaker of

cold 0.1 M ammonium formate (pH 3.0), once with 95% ethanol, and once with ether. [^{14}C]-adenosylmethionine was quantified by liquid scintillation counting of dried disks under 5 ml of Fisher Scint Verse II.

SAM synthetase was isolated by suspending 8 g of pelleted *L. mexicana* 227 cells in buffer containing 50 mM Tris (pH 7.5), 10 mM MSG_4, 1 mM EDTA, and 1 mM dithiothreitol. The cells were sonicated three times for 15 s each time, and the cell suspension was centrifuged at 4°C for 90 minutes at 40,000 X *g* in an SW 55 Ti rotor. The cell extract (5.3 ml) was applied to a DEAE-cellulose column, and the above buffer was passed through the column until the A_{280} was less than 0.1. the enzyme was then eluted with a linear gradient of KCl (0 to 0.3M) in a volume of 80 ml.

COMPOUND TESTED AGAINST LEISHMANIA MEXICANA 227 (IC_{50} uM)

	WR_NUM	Name	Date	Mode of Action	Approved for Human Use	IC50 (uM)	IC50 (ug/ml)	Max. % Inhib.	Highest Conc. Tested(uM)	Comments
65105	WR 240811 AA					184.00				
65141	WR 263527 AA					165.00				
64911	WR 183751 AA							26.6	570	
64920	WR 184362 AA							13.7	500	
65089	WR 221656 AA							34.3	1000	
65150	WR 249888 AA							27.1	670	
65169	WR 249909 AA							26.5	670	
65178	WR 249941 AA							35.6	1000	
64831	WR 153335 AA							0	400	
64840	WR 171304 AA							0	400	
64859	WR 171333 AA							0	400	
64877	WR 182971 AA							0	400	
64886	WR 182968 AA							0	400	
64902	WR 183750 AA							0	1000	
64957	WR 217246 AA							0	500	
64975	WR 218555 AA							0	375	
64984	WR 218421 AA							0	375	
64993	WR 218418 AA							0	500	
65007	WR 218413 AA							0	500	
65025	WR 220046 AA							0	500	
65034	WR 220033 AA							0	500	
65114	WR 040320 AB							0	1000	
65123	WR 244633 AB							0	250	
65187	WR 249940 AA							0	1000	
64895	WR 183119 AA							0	1000	stimulation
64939	WR 184358 AA							0	800	stimulation
64948	WR 185204 AA							0	1000	stimulation
64966	WR 218368 AA							0	1000	stimulation
65016	WR 219984 AA							0	500	stimulation, precipitate noted in assay tubes
65043	WR 220001 AA							0	1000	stimulation
65052	WR 221235 AA							0	1000	stimulation
65070	WR 222056 AA							0	1000	stimulation
65098	WR 230639 AA							0	1000	stimulation
65132	WR 249721 AA							0	500	stimulation, precipitate noted in assay tubes
	WR 2446							0	120	stimulation
		Azithromycin						25	1000	
		Azidothymidine						0	900	stimulation
		Dideoxycytidine						0	1000	

	WR_NUM	Name	Date	Mode of Action	Approved for Human Use	IC50 (uM)	IC50 (ug/ml)	Max. % Inhib.	Highest Conc. Tested(uM)	Comments
		Dideoxyinosine						0	1000	
		Pentamidine isethionate				42.00				
		Sulfamethoxazole						0	500	
		Sinefungin, Sigma 1				0.03				Sigma 1 - frozen sample, thawed
		Sinefungin, Sigma 2				0.24				Sigma 2 - Freshly prepared
58705	WR 254847 AB	Sinefungin, Walter Reed				0.21				
		Sinefungin, Calbiochem				0.24				
		Elephant garlic (protein extract)					12.5			
		Formycin B		RNA, protein synthesis	No			0	0.04	stimulation
		Cyclic Formycin A monophosphate		Unknown, Growth Inhibitor	No			0	0.06	stimulation
		Allopurinolriboside		RNA, protein synthesis	Now in trial			0	190	stimulation
		4-Aminopyrazolo-pyrimidine		RNA, protein synthesis	No			0	76	stimulation
		N^6-Methylaminopurine-9-ribofuranoside		Guanase, Adenine deaminase Nucleoside hydrolase Transport purines	No					6.5uM Ki for Guanase
		6-Methylaminopurine-9-ribofuranoside		Transport of Nucleosides	No	250.00				
		Hypoxanthine-9-beta-D-arabinofuranoside		Transport of Inosine	No	110.00				
		6-Methlypurine		Transport of Nucleosides	No	75.00				
		6-Methlyoxypurine-riboside		Transport of Adenosine	No	75.00				
		6-Mercaptopurine-riboside		Transport of Nucleosides	No	75.00				
		Adenosine, N^6-cyclohexyl		Transport of Adenosine	No	75.00				
		8-Phenyltheophylline		Transport of Nucleosides		75.00				
		2-Mercaptopyrimidine		Growth Inhibition	No	250.00				
		5-Flouorouracil		Growth Inhibition	Yes	300.00				
		4-Mercapto-2-pyrazolo [3,4-d] pyrimidine		Growth Inhibition	No	750.00				
		Arachidonic Acid				350.00				
		Eicoapentaenoic Acid				660.00				
		Linoleic Acid				600.00				
03027	WR 268613 AA	no name								insoluble, not tested
03009	WR 268612 AA	no name								insoluble, not tested
02904	WR 268603 AA	no name								insoluble, not tested
02931	WR 268605 AA	no name								insoluble, not tested
02959	WR 268607 AA	no name								insoluble, not tested
02986	WR 268610 AA	no name								insoluble, not tested
03045	WR 268614 AA	no name								insoluble, not tested
03054	WR 268615 AA	no name								insoluble, not tested
59588	WR 268317 AA	1,2-Dimethyl-3-hydroxypyrid-4-one				70.00				
21100	WR 243251 AC	7-Chloro-3-(2",4"-dichlorophenyl)-1-[[33'-(dimethlamino)propyl]imino]-1,2,3,4-tetrahydro-9(10H)acridinone				178 (mM)				
29759	WR 250548 AB	(S)-7-Chloro-3-(2,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-[[3-(dimethylamino)propyl]imino]-9-acridinol								insoluble, not tested
34170	WR 250547 AB	(R)-7-Chloro-3-(2,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-[[3-(dimethylamino)propyl]imino]-9-acridinol						16	150	

IN	WR_NUM	Name	Date	Mode of Action	Approved for Human Use		IC50 (uM)		IC50 (ug/ml)	Max. % InhIb.	Highest Conc. Tested(uM)		Comments
IL 56390	WR 182393 AB	1-(3,4-Dichlorophenyl)-3-(1-isopropyl-4,5-dioxo-2-imidezolidinylidine)-guanidine								8	292		
IM 02995	WR 268611 AA	no name											insoluble, not tested
IM 02997	WR 268602 AA	no name											insoluble, not tested
IM 02959	WR 268607 AA	no name											insoluble, not tested
IM 03083	WR 268616 AA	no name											insoluble, not tested
IM 03018	WR 257801 AB	no name											insoluble, not tested
IM 02998	WR 268601 AA	no name											to be tested
IM 02913	WR 268604 AA	no name											insoluble, not tested
M 02940	WR 268606 AA	no name											insoluble, not tested
M 02977	WR 268609 AA	no name											insoluble, not tested
M 08608										58.2	1000		test against higher concentration
K 73252	WR 236805 AC	8-[(4-Amino-1-methylbutyl)amino]-2,6-dimethoxy-4-methyl-5-(3-trifluoromethylphenoxy)quinoline succinate											compound binds and PPT media components
K 01845	WR 006026 AF	6-Methoxy-8-(6-diethylaminohexylamino)lepidine, dihydrochloride					1 (mM)						
M 04186	WR 242511 AD	8-[(4-Amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline DL-Tartarate					1.25 (mM)						
M 08175	WR 269097 AA	3-Deazaaristeromycin								22.3	1000		test against higher concentration
M 07918	WR 266970 AA	3-Deazaneplanocin,hydrochloride,hemihydrate								28.6	1000		test against higher concentration
B-1									25				tested against L. mex. 222 resulting in an IC50 of 25 ug/ml
B-3								<<	10				tested against L. mex. 222 result-ing in an IC50 of <<10
N-6e										32	50 ug/ml		
L-55										0	50 ug/ml		

COMPOUNDS TESTED AGAINST HUMAN CEM T_4 CELLS (IC_{50} uM)

BN	WR_NUM	Name		IC50 (uM)	IC50 (ug/ml)		IC25 (uM)	Comments
		Sinefungin		11000	28.8			
		DFMO		10400	57.1			Eflornithine (DL-alpha-difluoromethylornithine)
		Sulfamethoxazole		3900	16.7			
	WR 2446			2270	5.8			
		Naltrexone		1656	4.4			An Immune Modulator that stimulates CD4+ cells in vivo.
		Flucytosine	>	1500	11.6			
	WR 183750			1020	2.2			
		TMP-SMX		52.6	0.18			Trimethoprim - Sulfamethoxale (Proloprim or Bactrim) in 1:19 ratio.
		Acyclovir		540	2.4			Zovirax
		Dapsone	>	500	2			
		Trimethoprim		420	1.4			
		AZT		220	0.8			Zidovudine
		Pentamidine		7	0.012			
		2'3'-Dideoxycytidine		6	0.028			
		Ketoconazole		2.3	0.004			
BL 59588				460				
BL 21100				92				
BL 34170				26				
BL 56390				96				
		Allopurinol Riboside	>	12300				
		Meglumine antimoniate (Glucantime)	>	12000				Meglumine antimoniate
		9-deazainosine		4000				
		Cyclic sinefungin	>	3000				
		Cordycepin		3000				
		SIBA		250				5-deoxy-5(isobutylthio)-3-adenosine

BN	WR_NUM	Name		IC50 (uM)	IC50 (ug/ml)		IC25 (uM)	Comments
		Formycin B		13				
		SRTC		13				5'-o-sulfamoyl-1-B-D-ribofuranosyl triazole-3-carboxamide
		7-deazainosine		12				
		Formycin A	<	8				
		Allium sativus (Garlic)			11			11 ug protein/ml (0.001 of a clove/ml) This is made from a crude extract of raw garlic which is diluted in double-distilled water and sterile-filtered.
ZP 64831	WR 153335 AA		>	1000			390	
ZP 64840	WR 171304 AA			430			253	
ZP 64859	WR 171333 AA		>	1000		>	1000	
ZP 64877	WR 182971 AA			1004			325	
ZP 64886	WR 182968 AA			379			240	
ZP 64895	WR 183119 AA		>	1000		>	1000	
ZP 64902	WR 183750 AA		>	1000			317	
ZP 64911	WR 183751 AA			26			8	
ZP 64920	WR 184362 AA			927			259	
ZP 64939	WR 184358 AA		<	10		<	10	
ZP 64948	WR 185204 AA		>	1000		>	1000	
ZP 64957	WR 217246 AA			354			116	
ZP 64966	WR 218368 AA			218			81	
ZP 64975	WR 218555 AA			300			121	
ZP 64984	WR 218421 AA			927			510	
ZP 64993	WR 218418 AA		>	1000		>	1000	
ZP 65007	WR 218413 AA		>	1000			605	
ZP 65016	WR 219984 AA			422			10	
ZP 65025	WR 220048 AA			1000			44	
ZP 65034	WR 220033 AA			68			16	
ZP 65043	WR 220001 AA			388			10	
ZP 65052	WR 221235 AA			587			58	
ZP 65070	WR 222056 AA		>	1000		>	1000	
ZP 65089	WR 221656 AA			100			10	
ZP 65098	WR 230639 AA		>	500			50	
ZP 65105	WR 240811 AA		<	10		<	10	
ZP 65114	WR 040320 AB		>	1000		>	1000	
ZP 65123	WR 244633 AB		>	1000			410	

BN	WR_NUM	Name		IC50 (uM)	IC50 (ug/ml)		IC25 (uM)	Comments
ZP 65132	WR 249721 AA			95			28	
ZP 65141	WR 263527 AA		>	1000		>	1000	
ZP 65150	WR 249868 AA			851			373	
ZP 65169	WR 249909 AA			309			133	
ZP 65178	WR 249941 AA			546			218	
ZP 65187	WR 249940 AA		>	1000			529	
		7-Deazaaristeomycin		19.6				
		Cyclic Formycin A		250				
		Oxyformycin B		2000				
		Sangivamycin		6.4				
		Spermidine	<	100				
HB-1								25% inhib. at 5 ug/ml
HB-3								21% inhib. at 5 ug/ml
PN-6a								22% inhib. at 5 ug/ml
DL-55								74% inhib. at 5 ug/ml

The Toxicity of 34 Adenosine Analogs to Human CD4 T-Lymphocytes as measured in ug/ml.

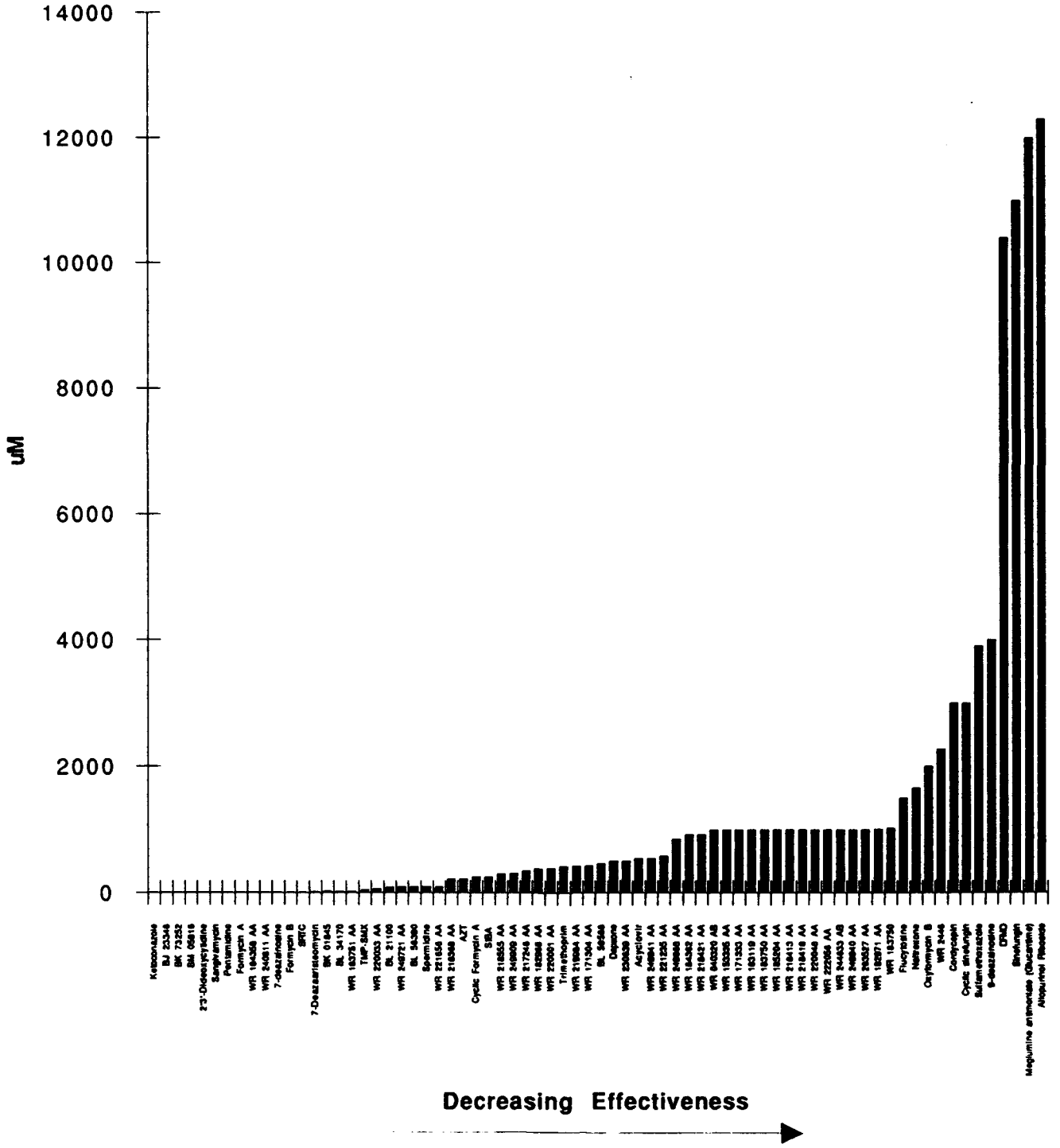
Compound: ZP Number	WR Number	ID_{50} (ug/ml)	ID_{25} (ug/ml)
ZP 64939	WR 184358 AA	<4	<4
ZP 65105	WR 240811 AA	<4	<4
ZP 64911	WR 183751 AA	10	3
ZP 65034	WR 220033 AA	25	6
ZP 65089	WR 221656 AA	34	3
ZP 65132	WR 249721 AA	43	13
ZP 64966	WR 218368 AA	77	28
ZP 64975	WR 218555 AA	121	44
ZP 64886	WR 182968 AA	122	77
ZP 65043	WR 220001 AA	131	3
ZP 65169	WR 249909 AA	131	56
ZP 64840	WR 171304 AA	145	85
ZP 64957	WR 217246 AA	163	53
ZP 65016	WR 219984 AA	181	4
ZP 65052	WR 221235 AA	222	22
ZP 65178	WR 249941 AA	227	91
ZP 64831	WR 153335 AA	>214	83
ZP 65098	WR 230639 AA	336	17
ZP 64877	WR 182971 AA	347	112
ZP 64920	WR 184362 AA	352	98
ZP 65150	WR 249868 AA	353	155
ZP 65187	WR 249940 AA	358	189
ZP 65025	WR 220048 AA	371	16
ZP 65123	WR 244633 AB	388	159
ZP 65007	WR 218413 AA	>326	197
ZP 64902	WR 183750 AA	>470	149
ZP 64984	WR 218421 AA	510	143
ZP 64859	WR 171333 AA	>295	>295
ZP 64993	WR 218418 AA	>303	>303
ZP 64895	WR 183119 AA	>320	>320
ZP 64948	WR 185204 AA	>341	>341
ZP 65070	WR 222056 AA	>343	>343
ZP 65114	WR 040320 AB	>354	>354
ZP 65141	WR 263527 AA	>374	>374

Enzyme Studies on DNA Polymerases from *L. mexicana* 227

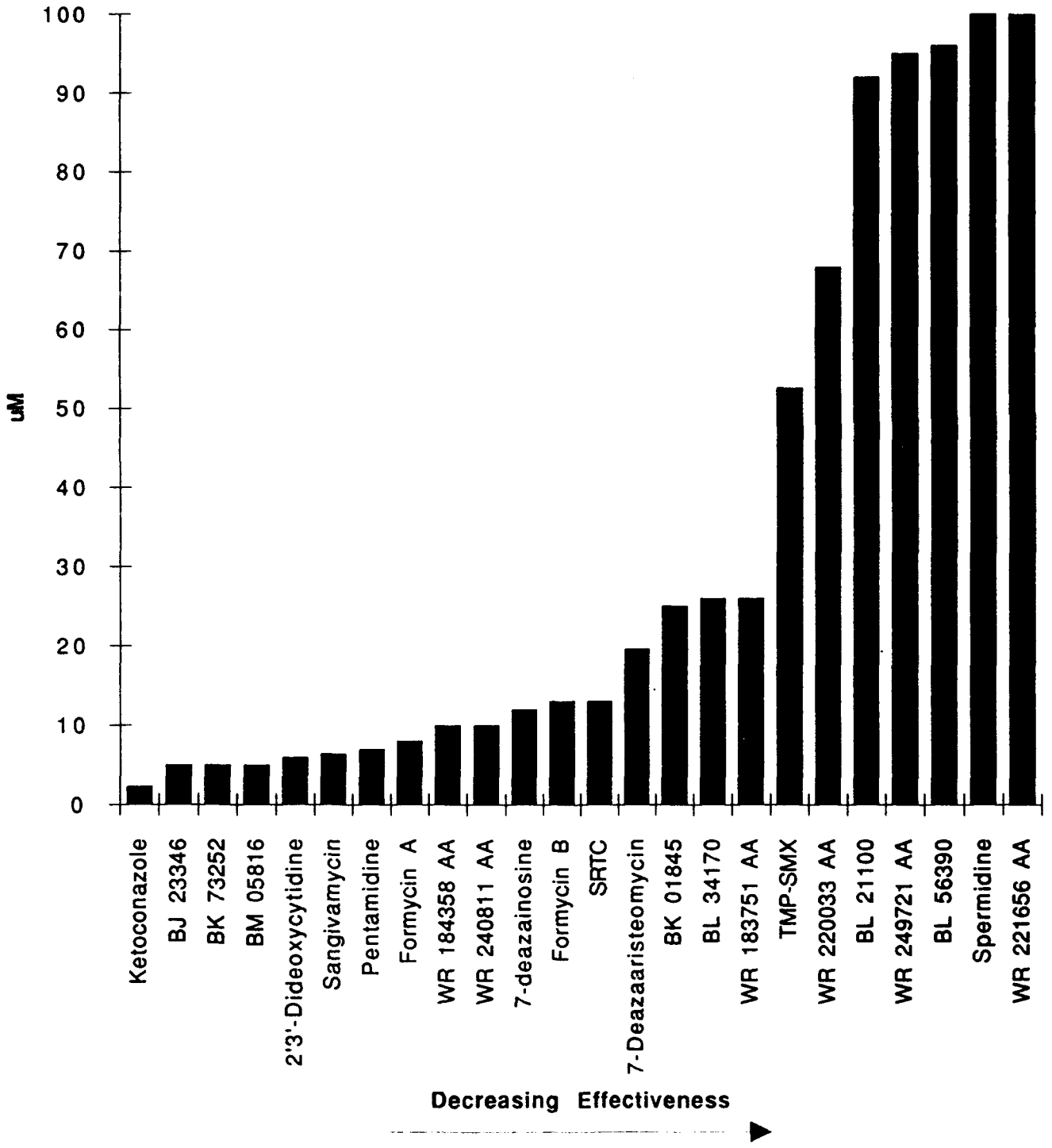
					A Polymerase				B Polymerase			
					Active	Inactive Compounds			Active	Inactive Compounds		
Bottle No.	WR No.	Name	Approved for Human Use		IC50	Max. % Inhib.	Highest Conc. Tested(uM)		IC50	Max. % Inhib.	Highest Conc. Tested(uM)	Comments
		Ara CTP				0	500	>	500		500	
		Aphidicolin				6	400			2	400	
		Ethidium Bromide		<	6				29			
		N-Ethylmaleimide		<	1000				1000			A Enzyme inhibited 94% by 5mM NEM, B Enzyme inhibited 86% by 5mM NEM
		Acyclovir				0	100			0	100	
		AZT				0	100			0	500	
	WR 2446					0	100			0	100	
	WR 783750					0	100			42	100	
		Pentamidine			100				NOT TESTED			
ZP 65105						33	500			0	500	
		dideoxy Inosine				21	500		NOT TESTED			
		dideoxy Cytosine				28	500		NOT TESTED			
		ddTTP				3	500		7.5			
		Spermine			250					0	500	
		Phosphomycin			1000				NOT TESTED			
		Azythromycin			500				NOT TESTED			
		Garlic Extract				17	1 (w/v)		NOT TESTED			
		Sinefungin				21	500		NOT TESTED			
		Oridion				30	200		NOT TESTED			
		Ponicidin				26	200		NOT TESTED			
		Cytosine 5' Carboxilic Acid				0	1000		NOT TESTED			
		Dapsone				12	33		NOT TESTED			
		Ketoconazole				11	10		NOT TESTED			
		Mitomycin C				11	2		NOT TESTED			
		Nalidixic Acid				15	10		NOT TESTED			
		Novobiocin				18	20		NOT TESTED			
		Sulfamethoxale				10	20		NOT TESTED			
		Trimethoprim				12	500		NOT TESTED			
		DFMO				0	2500		NOT TESTED			
		Berenil			40				50			
		BuPd GTP			25				5.4			
		BuAd ATP			150				50			
		Hemin			50				30			
		Suramin			7				2			
		Phosphonoacetic Acid			1500					0	2000	
		FPAA			130					0	160	
		BrPAA				0	800			0	800	
		ClPAA				0	800			0	800	
		F2PAA				10	2000			0	2000	

					A Polymerase				B Polymerase			
					Active	Inactive Compounds			Active	Inactive Compounds		
Bottle No.	WR No.	Name	Approved for Human Use		IC50	Max. % Inhib.	Highest Conc. Tested(uM)		IC50	Max. % Inhib.	Highest Conc. Tested(uM)	Comments
		COMDP				150	1000			0	1000	
		Arachidonic Acid			23-50*				40			*dependent on units of enzyme present
		Linoleic Acid			40				50			
		gamma-Linolenic Acid			40				40			
		Eicosapentaenoic Acid			140				207			
		Docohexaenoic Acid			150				192			

COMPOUNDS TESTED AGAINST HUMAN CEM T4 CELLS (IC50 uM)



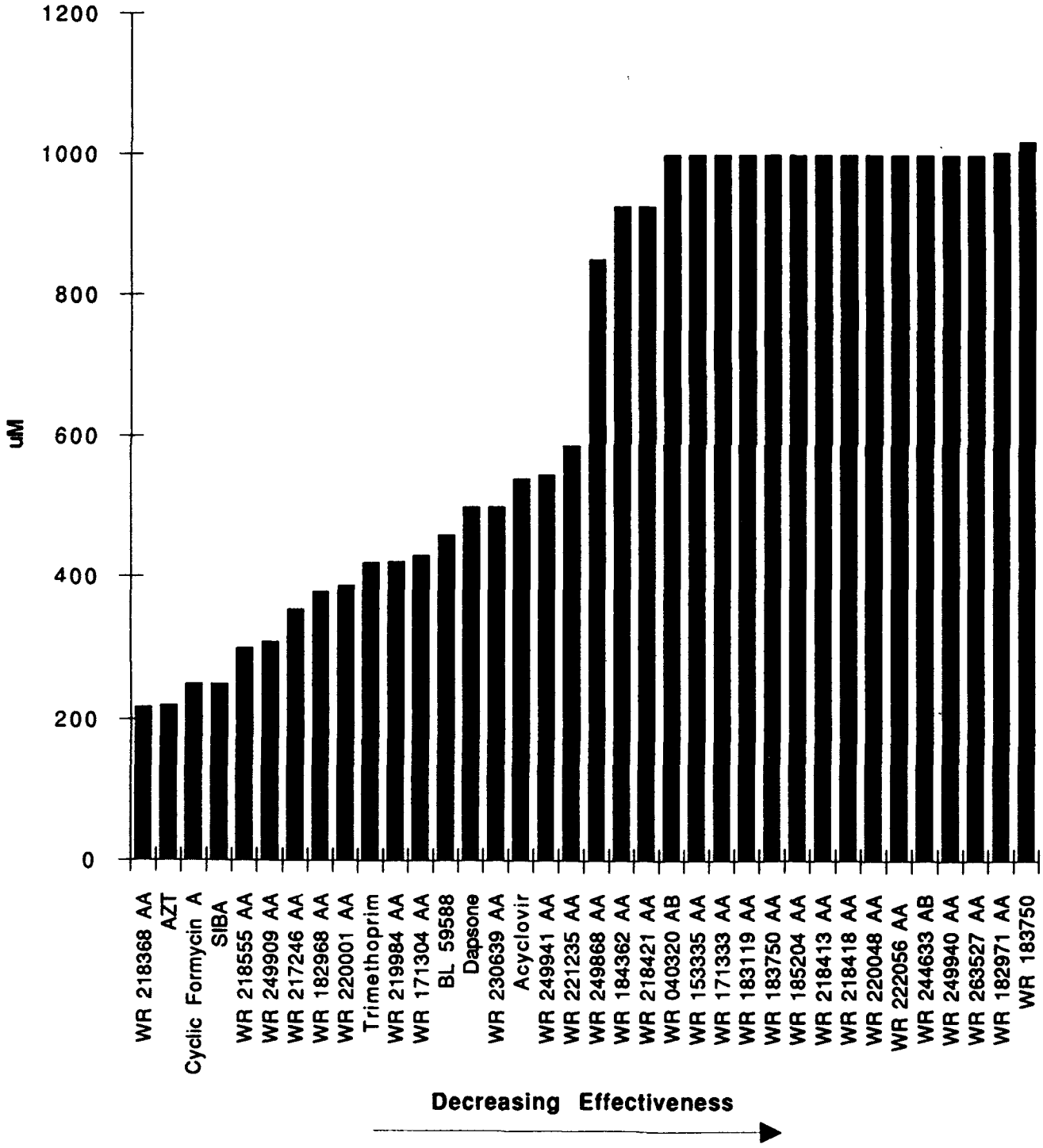
Very Effective Compounds for T4 Cells (IC50, uM)



Very Effective Compounds for T4 Cells (IC50, uM)

Compound	IC50, uM
Ketoconazole	2.3
BJ 23346	5
BK 73252	5
BM 05816	5
2'3'-Dideoxycytidine	6
Sangivamycin	6.4
Pentamidine	7
Formycin A	8
WR 184358 AA	10
WR 240811 AA	10
7-deazainosine	12
Formycin B	13
SRTC	13
7-Deazaaristeomycin	19.6
BK 01845	25
BL 34170	26
WR 183751 AA	26
TMP-SMX	52.6
WR 220033 AA	68
BL 21100	92
WR 249721 AA	95
BL 56390	96
Spermidine	100
WR 221656 AA	100

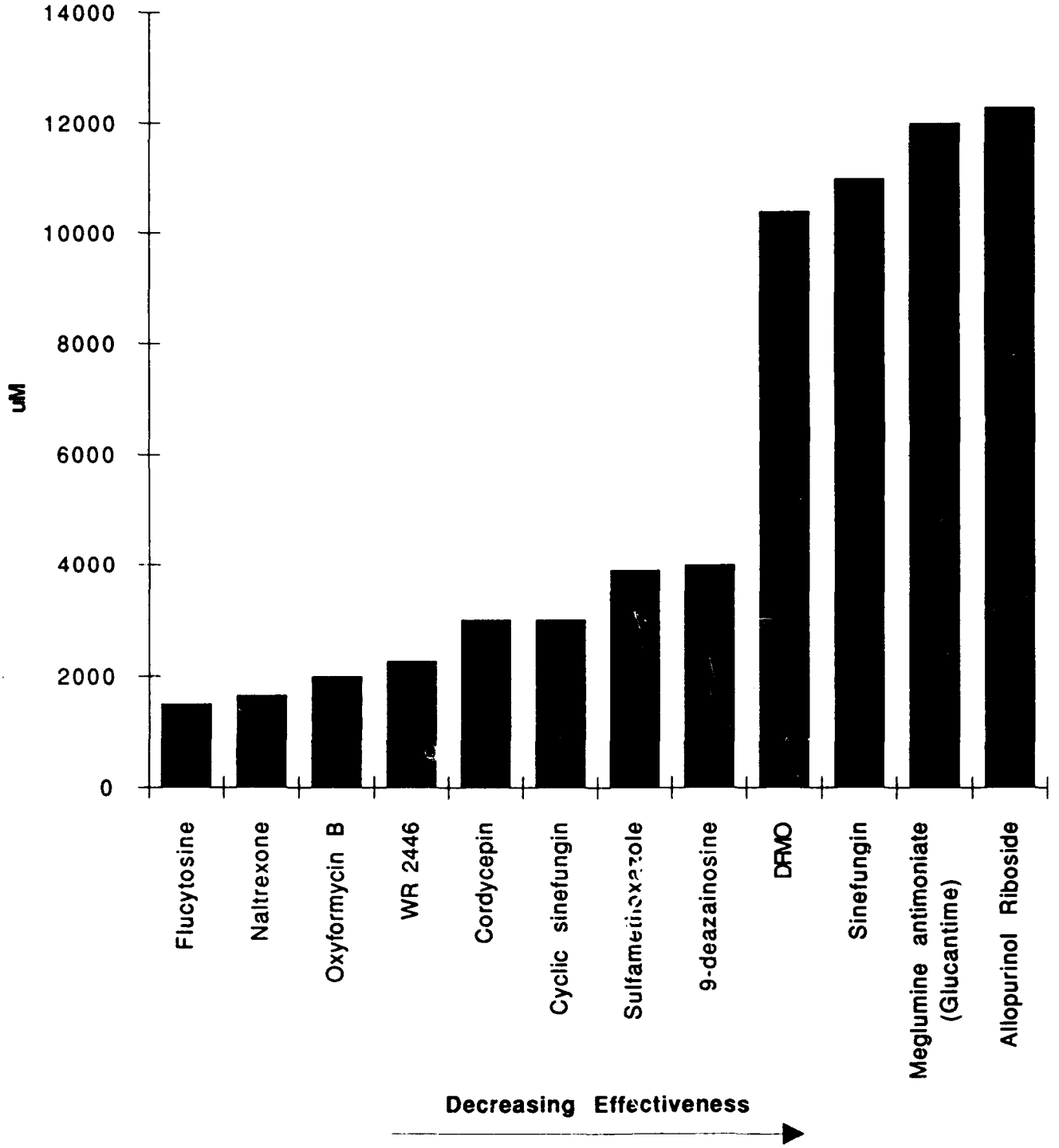
Moderately Effective Compunds for T4 Cells (IC50, uM)



Moderately Effective Compounds for T4 Cells (IC50, uM)

Compound	IC50, uM
WR 218368 AA	218
AZT	220
Cyclic Formycin A	250
SIBA	250
WR 218555 AA	300
WR 249909 AA	309
WR 217246 AA	354
WR 182968 AA	379
WR 220001 AA	388
Trimethoprim	420
WR 219984 AA	422
WR 171304 AA	430
BL 59588	460
Dapsone	500
WR 230639 AA	500
Acyclovir	540
WR 249941 AA	546
WR 221235 AA	587
WR 249868 AA	851
WR 184362 AA	927
WR 218421 AA	927
WR 040320 AB	1000
WR 153335 AA	1000
WR 171333 AA	1000
WR 183119 AA	1000
WR 183750 AA	1000
WR 185204 AA	1000
WR 218413 AA	1000
WR 218418 AA	1000
WR 220048 AA	1000
WR 222056 AA	1000
WR 244633 AB	1000
WR 249940 AA	1000
WR 263527 AA	1000
WR 182971 AA	1004
WR 183750	1020

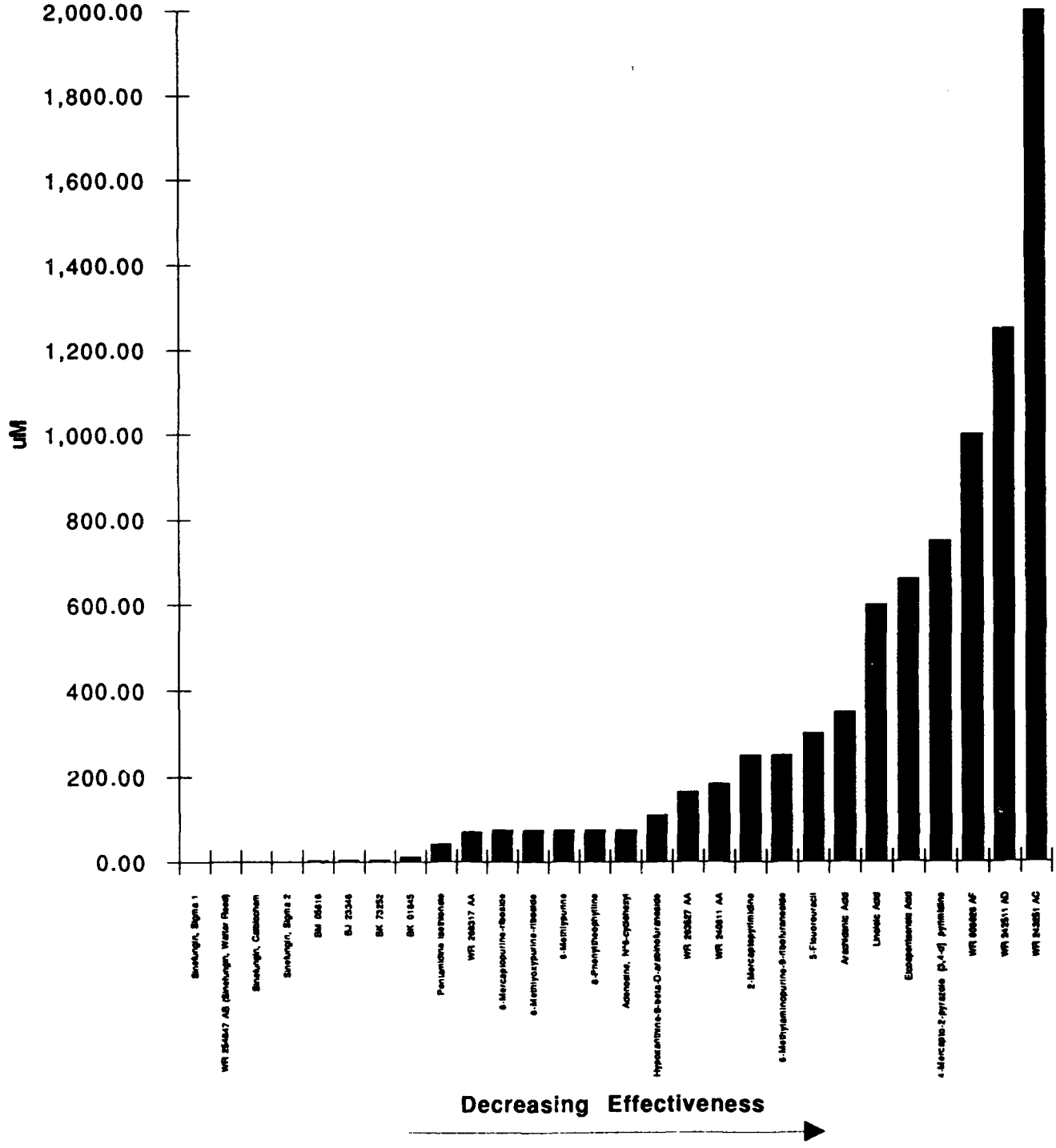
Least Effective Compounds for T4 Cells (IC50, uM)



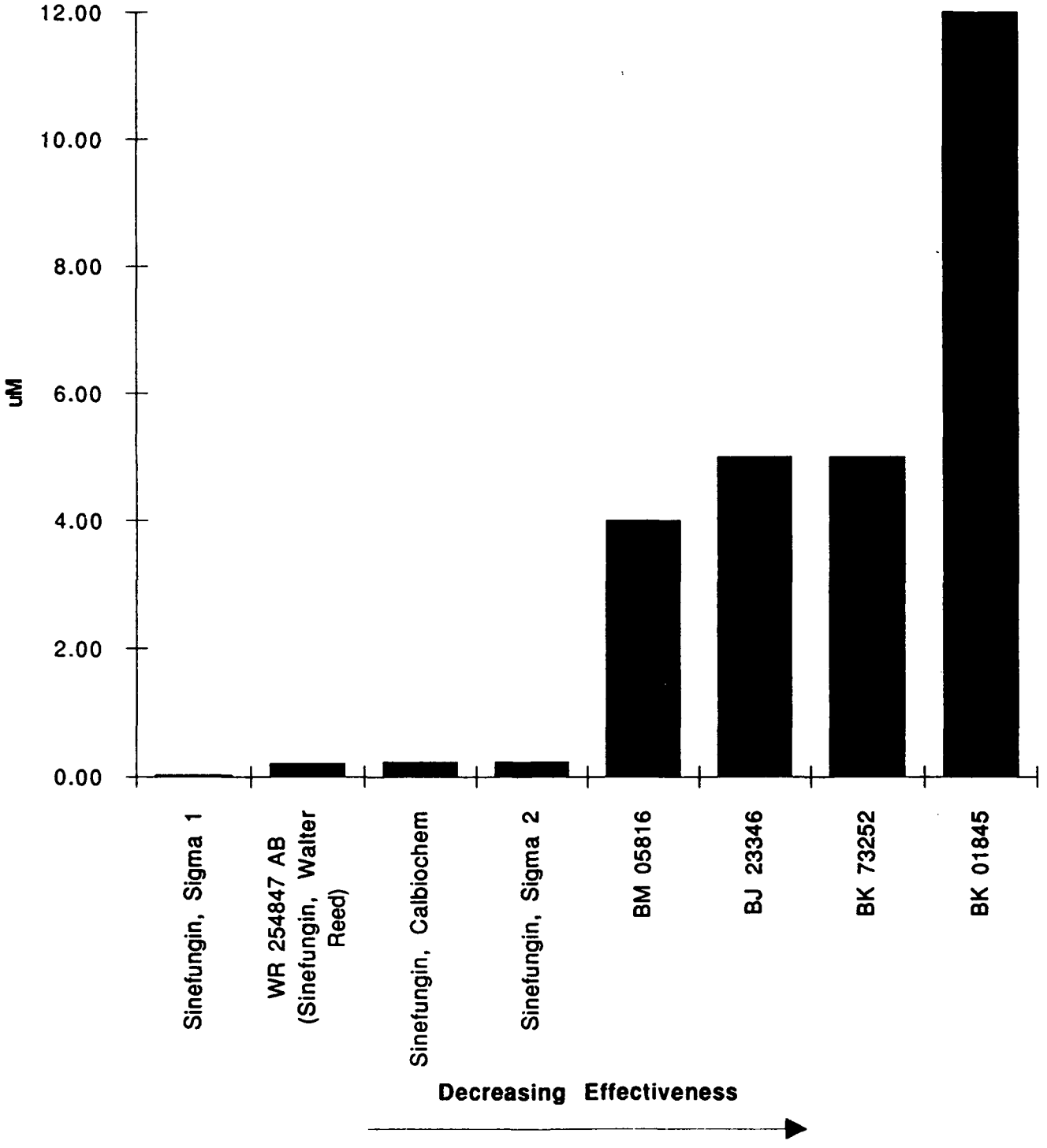
Least Effective Compounds for T4 Cells (IC50, uM)

Compound	IC50, uM
Flucytosine	1500
Naltrexone	1656
Oxyformycin B	2000
WR 2446	2270
Cordycepin	3000
Cyclic sinefungin	3000
Sulfamethoxazole	3900
9-deazainosine	4000
DFMO	10400
Sinefungin	11000
Meglumine antimoniate (Glucantime)	12000
Allopurinol Riboside	12300

Compounds for L. Mexicana (IC50, uM)



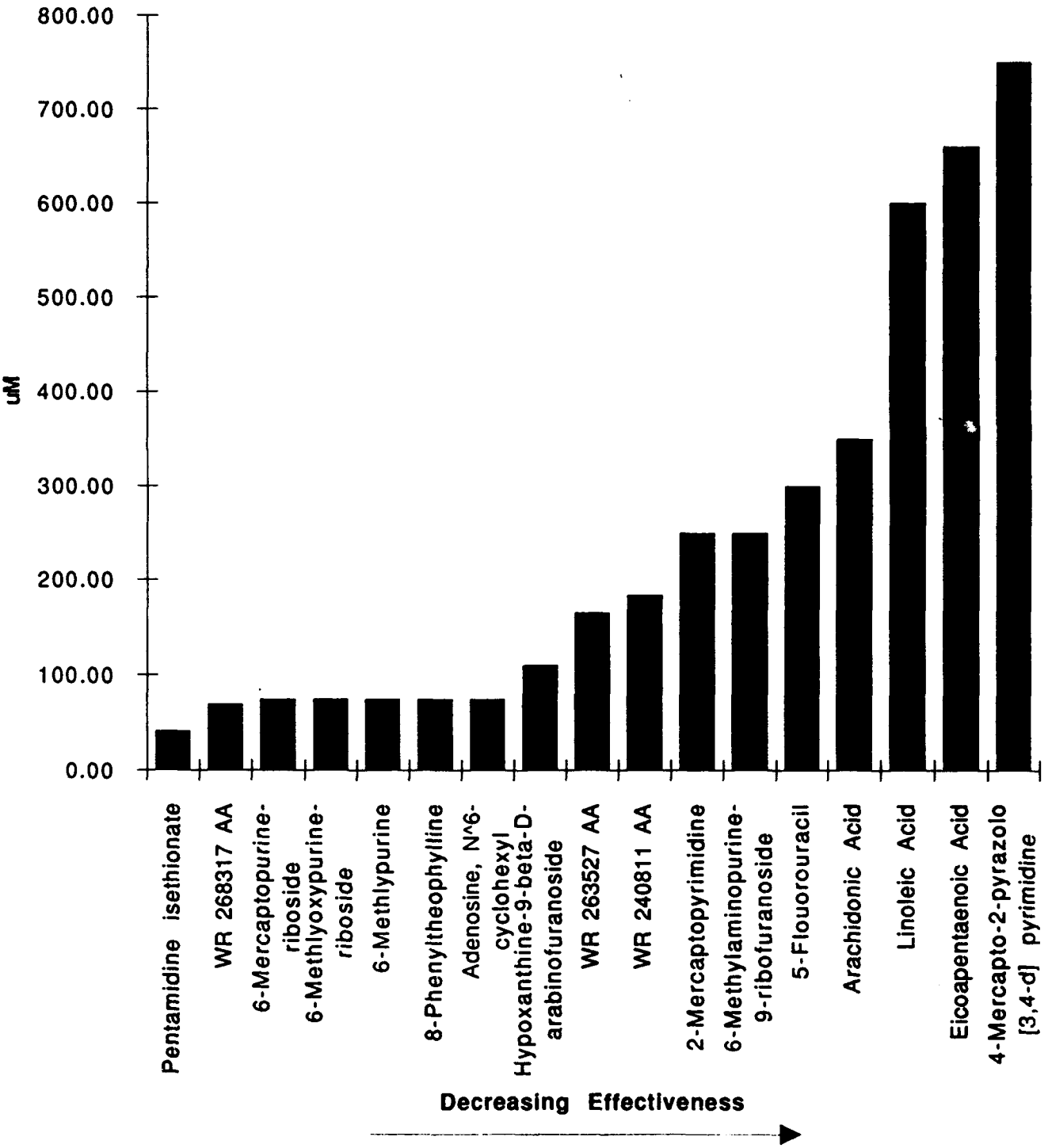
Very Effective Compounds for L. Mexicana (IC50, uM)



Very Effective Compounds for L. Mexicana

Compound	IC50, uM
Sinefungin, Sigma 1	0.03
WR 254847 AB (Sinefungin, Walter Reed)	0.21
Sinefungin, Calbiochem	0.24
Sinefungin, Sigma 2	0.24
BM 05816	4.00
BJ 23346	5.00
BK 73252	5.00
BK 01845	12.00

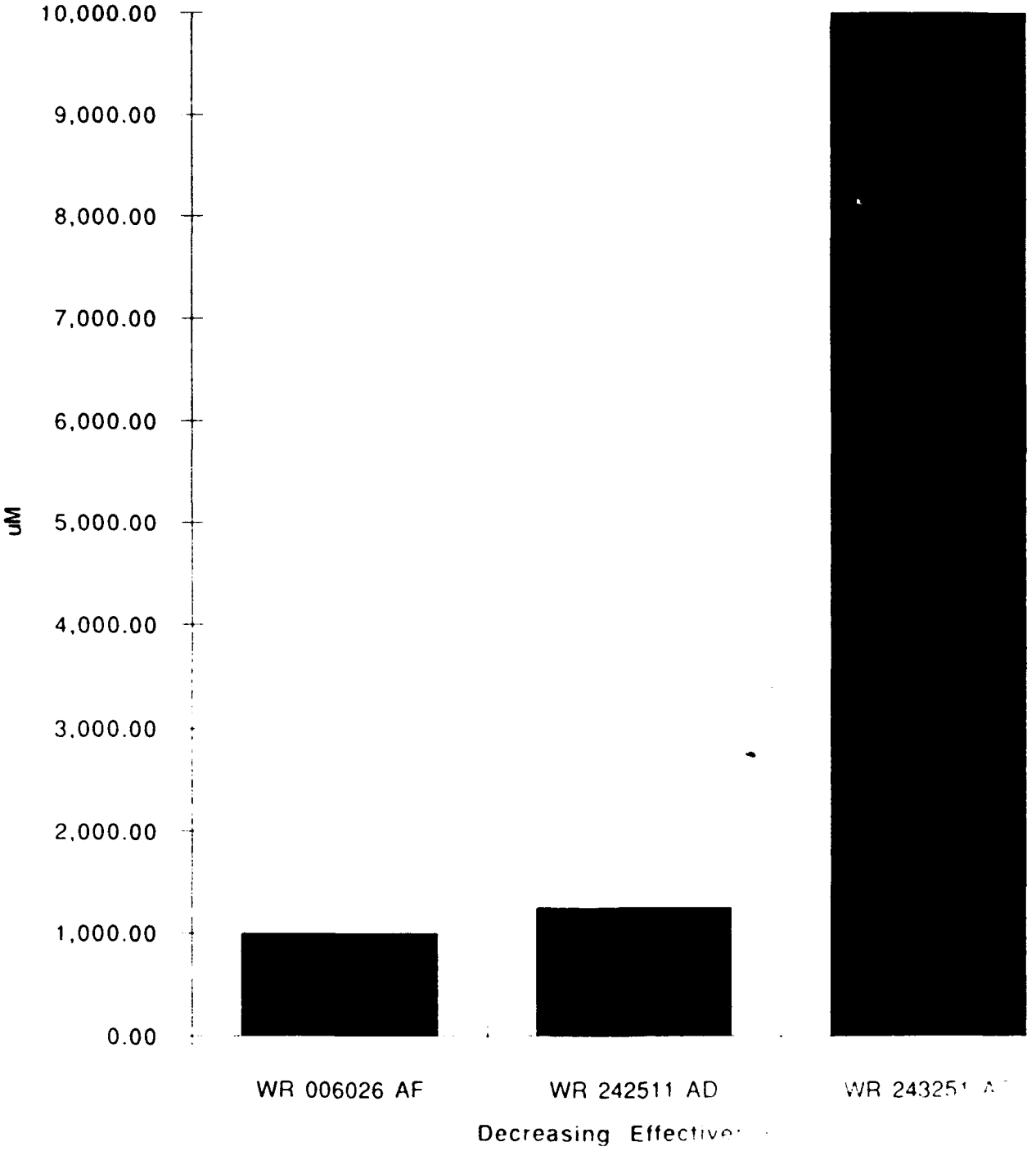
Moderately Effective Compounds for L. Mexicana (IC50, uM)



Moderately Effective Compounds for L. Mexicana

Compound	IC50, uM
Pentamidine isethionate	42.00
WR 268317 AA	70.00
6-Mercaptopurine-riboside	75.00
6-Methlyoxypurine-riboside	75.00
6-Methlypurine	75.00
8-Phenyltheophylline	75.00
Adenosine, N^6-cyclohexyl	75.00
Hypoxanthine-9-beta-D-arabinofuranoside	110.00
WR 263527 AA	165.00
WR 240811 AA	184.00
2-Mercaptopyrimidine	250.00
6-Methylaminopurine-9-ribofuranoside	250.00
5-Flouorouracil	300.00
Arachidonic Acid	350.00
Linoleic Acid	600.00
Eicoapentaenoic Acid	660.00
4-Mercapto-2-pyrazolo [3,4-d] pyrimidine	750.00

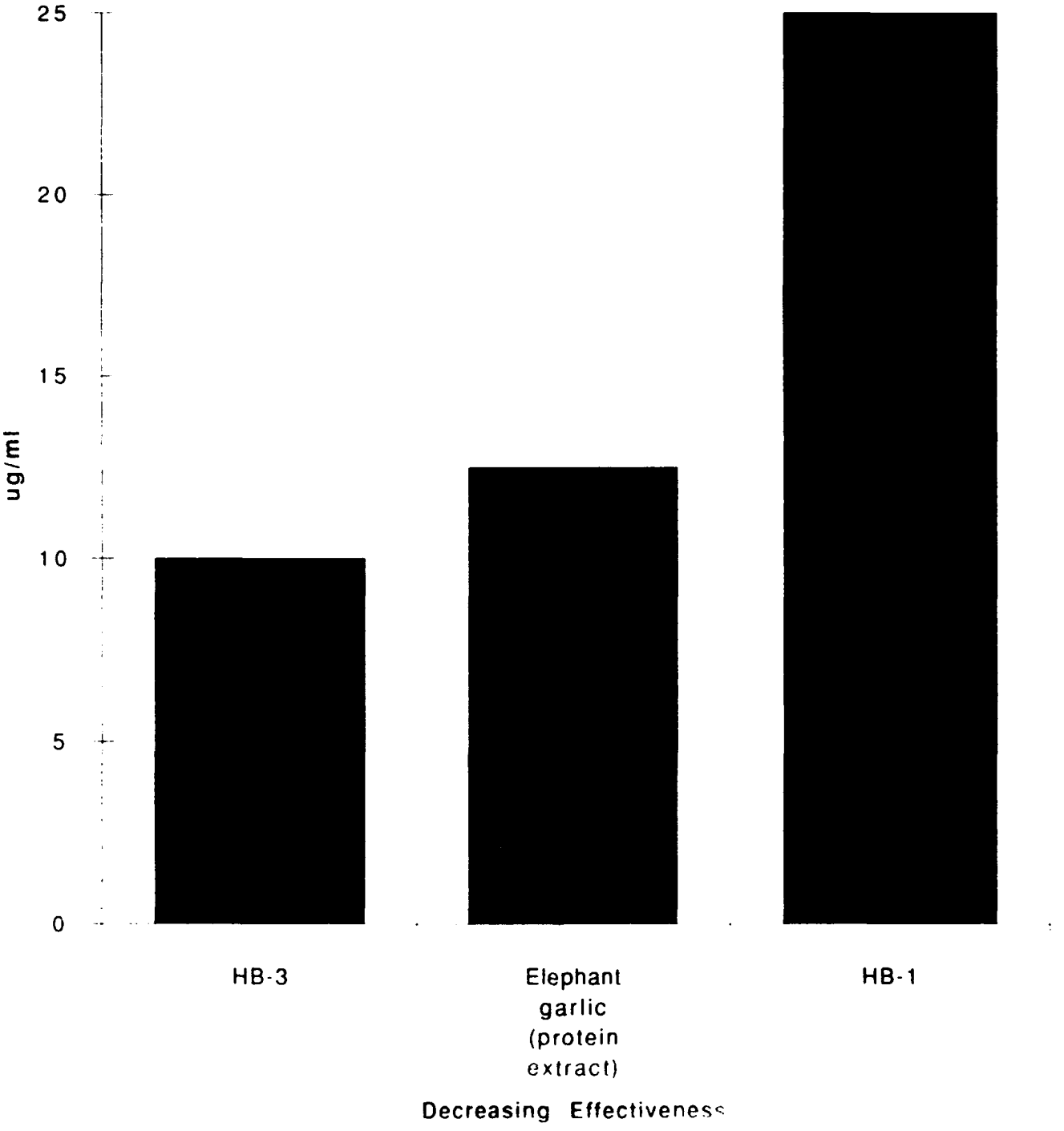
Least Effective Compounds for L. Mexicana (IC50, uM)



Least Effective Compounds for L. Mexicana

Compound	IC50, uM
WR 006026 AF	1,000.00
WR 242511 AD	1,250.00
WR 243251 AC	178,000.00

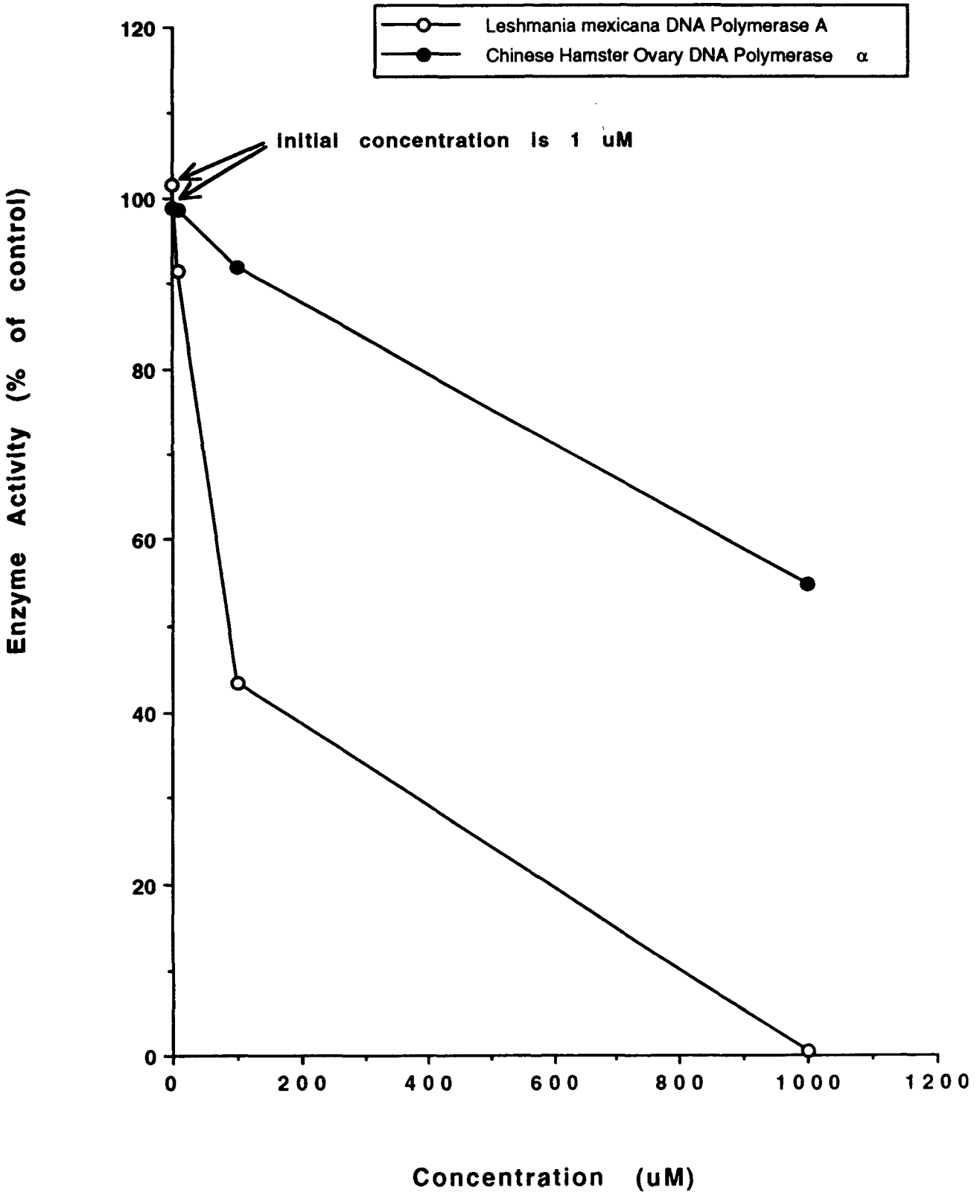
Natural Compounds for L. Mexicana 222 (IC50, ug/ml)



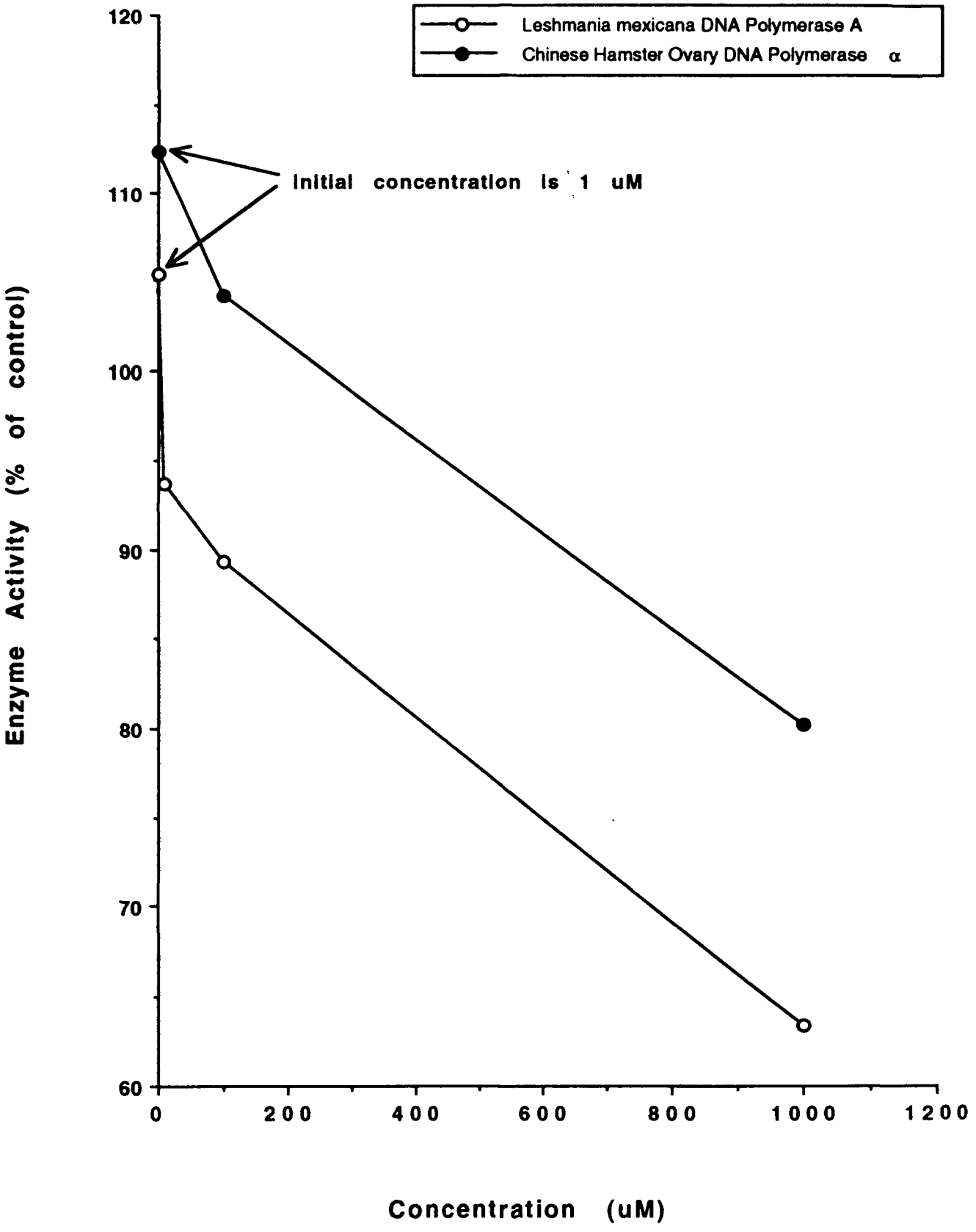
Natural Compounds for L. Mexicana 222

Compound	IC50, ug/ml
HB-3	10.00
Elephant garlic (protein extract)	12.50
HB-1	25.00

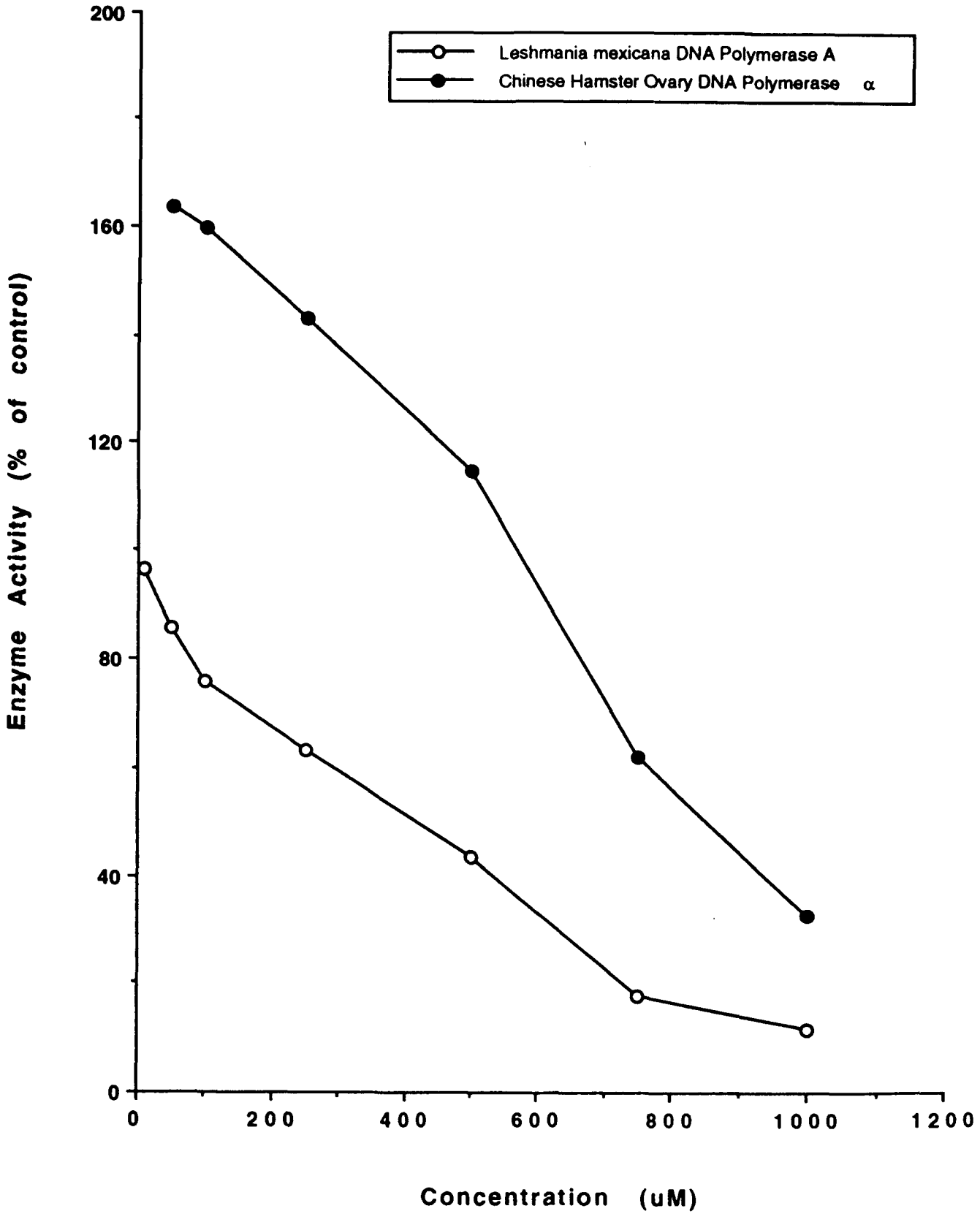
WRAIR Compound BJ 23346 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase α



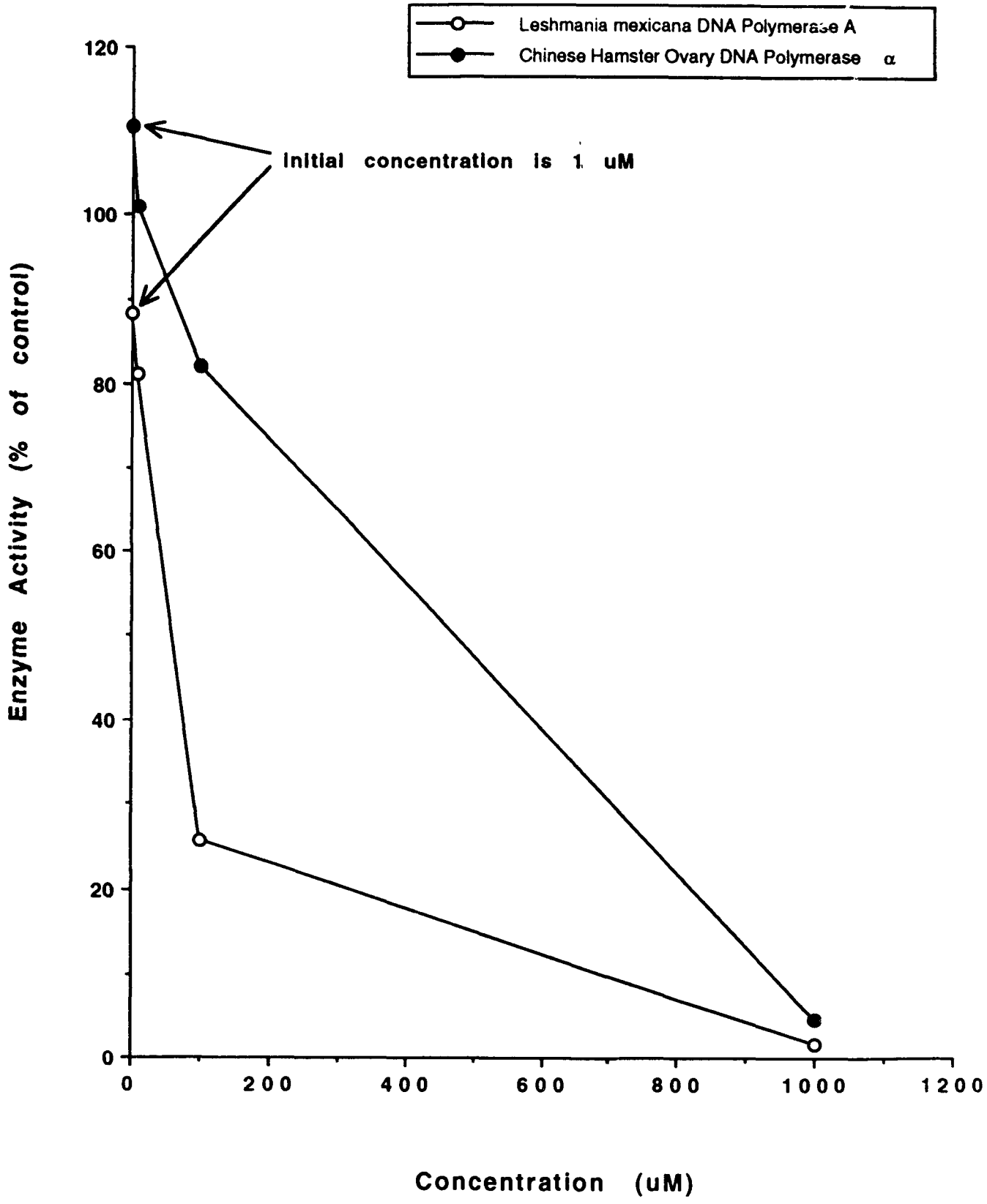
WRAIR Compound BK 01845 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase α



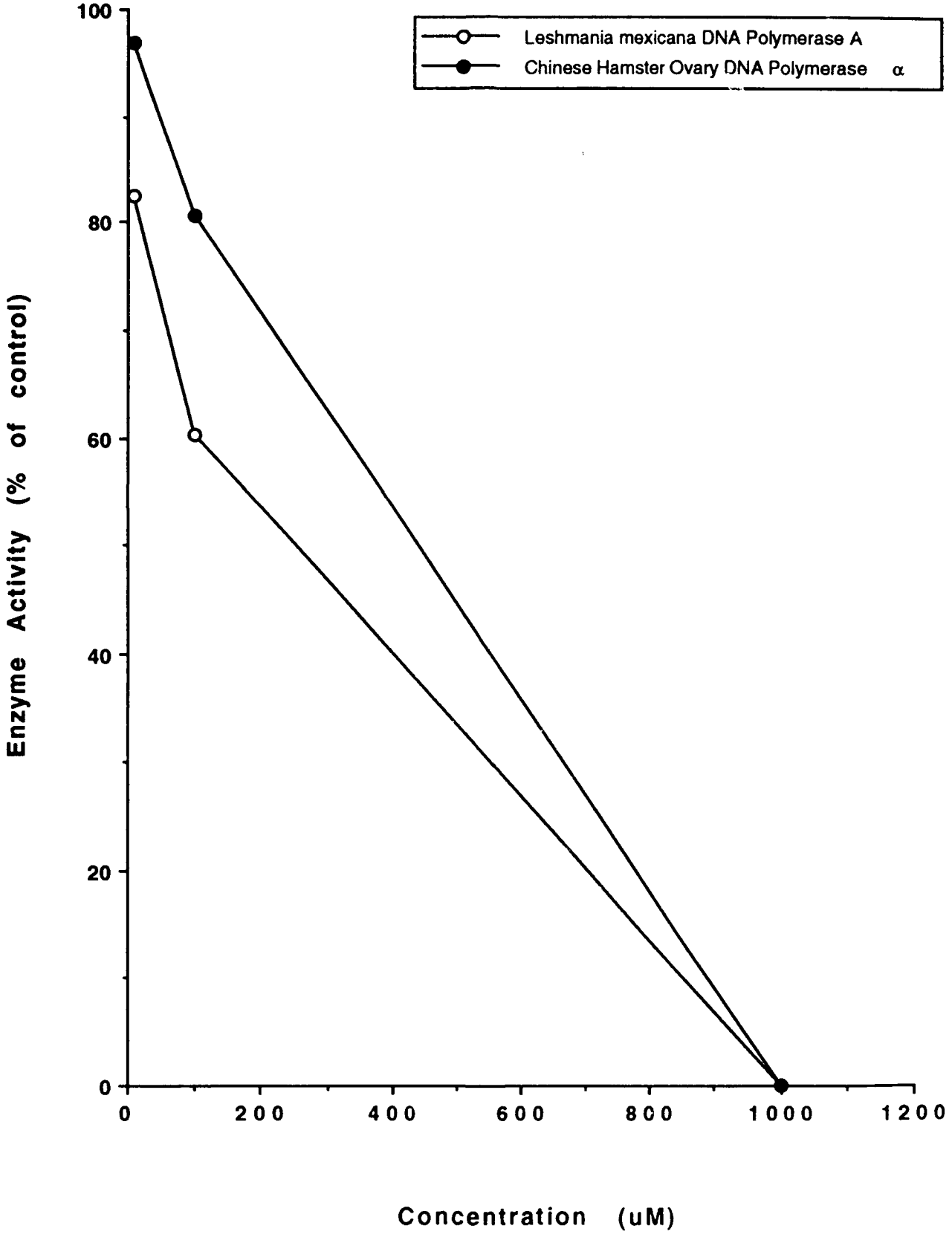
WRAIR Compound BK 40735 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase



WRAIR Compound BK 73252 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase



WRAIR Compound BM 05816 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase



THE DNA POLYMERASES OF *LEISHMANIA MEXICANA*

SUMMARY

Two previously isolated DNA polymerases from the parasitic protozoan *Leishmania mexicana* were further characterized by exposure to inhibitors of mammalian DNA polymerases. DNA polymerase A, a high molecular weight enzyme, and DNA polymerase B, a b-like DNA polymerase were compared to each other and to their mammalian counterparts regarding pH optimum, utilization of templates, and response to various inhibitors and ionic strengths. The results suggest the DNA polymerases from *L. mexicana* differ from the host enzymes and may offer a target for chemotherapeutic intervention.

INTRODUCTION

Five classes of DNA polymerase (a, b, c, d and e) have been isolated from higher eukaryotic cells (5-9) and a, b and c-like polymerases from parasitic protozoa (1-18). DNA polymerases a, d and e are nuclear enzymes associated with chromosomal replication; b is a low molecular weight nuclear enzyme involved in DNA repair (2,15,16), and c which has been isolated from mitochondria is believed to be responsible for mitochondrial DNA replication (6 9 21).

We have been studying DNA replication in the kinetoplast parasite *Leishmania mexicana* and have begun studies to characterize the major polymerase activities in these parasites for the purpose of comparing

them to host polymerases, particularly a and b. Although North and Wyler reported studies of *in vivo* DNA replication of *Leishmania* parasites (22), this laboratory is the first to report the isolation and characterization of the leishmanial DNA polymerases in vitro (10-12). Others have described purification of a-like, b-like (13)and c-like polymerases (18) from the parasitic protozoans *Crithidia fasciculata* and an a-like polymerase from *Trypanosoma brucei* (14) and *Trypanosoma cruzi* (15).

The purpose of this study is to compare the major DNA polymerase activities (A and B) isolated from *Leishmania mexicana* to a and b polymerases isolated from other sources.

MATERIALS AND METHODS

Test organism

Leishmania mexicana amazonensis (Walter Reed strain 227) obtained from the Leishmania section of the Walter Reed Army Institute of Research were grown in brain heart infusion media as previously described (10).

Preparation of Compounds

Aphidicolin (Sigma Chemical Co., St. Louis, MO) was prepared in dimethylsulfoxide (DMSO) as a 5 mM stock and diluted with water so that the final concentration of DMSO in the assay was no more than 0.16% (v/v). Suramin, purchased from Miles Pharmaceuticals (West Haven, CT), was made into a 70 mM stock solution in 10 mM Tris pH 7.5. Further dilutions were made with the same buffer. Butylphenyl dGTP (BuPdGTP), carbonyldiphosphonate (COMDP) and the phosphonoacetic acid derivatives

BrPAA, ClPAA, FPAA, and F_2PAA were generous gifts from Dr. G. Wright, University of Massachusetts Medical Center (Worcester, MA), and were prepared in aqueous solution at the appropriate concentrations. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of DNA polymerases

DNA polymerase A, a high molecular weight DNA polymerase sensitive to N-ethylmaleimide (NEM) was isolated from *L. mexicana* promastigotes as described (11) A low molecular weight DNA polymerase classified as a b-like enzyme was isolated from promastigotes as described (12) This enzyme will be referred to as DNA polymerase B, to distinguish it from mammalian enzymes and to follow the designation used by Holmes et al. (13) for the *Crithidia fasciculata* DNA polymerases.

Drug assays

The inhibitory properties of several compounds were determined by pre-incubating the enzyme and drug in the assay mix. In order to characterize the enzymes, selective inhibitors of mammalian DNA polymerases were tested against both enzymes. DNA polymerase A was assayed at 35°C as described (11). DNA polymerase B activity was measured at 35°C as previously described (12).

RESULTS AND DISCUSSION

Isolation of DNA polymerases

Two types of DNA polymerase activity were separated using affinity chromatography with denatured DNA cellulose. The two enzyme activities were designated as DNA polymerase A (11) and a b-like DNA polymerase (12)

according to their molecular weight, pH optimum and response to *N*-ethylmaleimide.

The B enzyme (pol B) was less stable than the DNA polymerase A (pol A) at all stages of the purification. The use of a mixture of protease inhibitors as well as glycerol during the isolation procedures was essential for stability of the DNA polymerases. In addition, the use of a freshly prepared assay mix was critical in obtaining pol B enzyme activity. Difficulty in detecting a low molecular weight DNA polymerase in parasitic protozoans has resulted in conflicting reports from some groups regarding the presence of a low molecular weight DNA polymerase in *Trypanosoma brucei* (9 14). In addition, studies on *T. cruzi* detected only one DNA polymerase of high MW and no b-like enzymes (15).

Proper characterization of the DNA polymerases from *L. mexicana* is essential in order to compare them with the host enzymes, a first step in a strategy to develop chemotherapeutic agents. To date, all enzymes isolated from parasitic protozoans have been found to share some, but not all, of the characteristics of the mammalian enzymes (9 11-18).

Characterization Studies

Pol A was slightly stimulated by NaCl or KCl at concentrations of less than 15 mM, but rapidly inactivated by higher concentrations of salt (11). DNA polymerase B was slightly stimulated by 5 mM KCl only, but was more resistant to inactivation by higher concentrations of NaCl or KCl, with 35% of the activity remaining in the presence of 200 mM NaCl and 43% of the activity remaining in the presence of 200 mM KCl (12) Mammalian DNA polymerase a is inhibited by high ($\geq$ 100mM)

concentrations of salt, whereas DNA polymerases b and d are stimulated by such concentrations (6).

The optimum pH of the pol A enzyme is mildly acidic to neutral at 6.7, whereas the optimum pH of the pol B enzyme is basic, at 9.0. Table 1 shows a comparison of the activity of the enzymes with several template-primers. The pol A had a template preference for activated DNA and used poly(dA)·oligo$(dT)_{12-18}$ equally as well, with only 60% of the activity when poly(dC)·oligo$(dT)_{12-18}$ was the template (11). Pol B showed a six-fold preference for poly(dC)·oligo$(dT)_{12-18}$ as the template over activated DNA (8). The preferred template for DNA polymerases a and b is activated DNA, whereas the mitochondrial DNA polymerase is more active with poly(rA)·Oligo(dT). A notable point is the inability of pol B to utilize Mn^{+2} as the divalent cation activator. In contrast, mammalian DNA polymerase b is capable of using both Mn^{+2} and Mg^{+2} (6).

Inhibitor Studies

Exposure of the enzymes to specific DNA polymerase inhibitors showed the *L. mexicana* enzymes to be different from one another and from mammalian enzymes in their sensitivity to various compounds (Table 2). Both enzymes were resistant to aphidicolin, a mammalian DNA polymerases a, d and e inhibitor. The response of these enzymes to the mammalian DNA polymerase a inhibitor BuPdGTP was interesting. In the presence of 100 lM dGTP, Pol B was twenty fold more sensitive to this compound than Pol A with a concentration that inhibits activity by 50% (IC_{50}) of 5.4 lM, whereas the Pol A was inhibited with an IC_{50} of 100 lM. Phosphonoacetic acid (PAA) was a weak inhibitor of the Pol A with only 35% inhibition at 2 mM. Pol B was resistant to PAA at concentrations of up to 2 mM. Mammalian b polymerase has been found to be resistant to

inhibition by this compound (6). Several PAA analogues (25) were tested against the *L. mexicana* DNA polymerases (Table 2). Pol B was completely resistant to inhibition by the fluoro, bromo, chloro, and difluoro analogues of PAA (FPAA, BrPAA, ClPAA, F_2PAA; respectively). Pol A was resistant to BrPAA, ClPAA, and F_2PAA. FPAA, a monohalogenated derivative of PAA, inhibited pol A with an IC_{50} of 130 1M, resulting in over a ten fold increase in inhibition compared to PAA. FPAA also exhibited potent inhibition of the calf thymus DNA polymerases a and d [Table 2; (25;]. COMDP, a specific inhibitor of mammalian DNA polymerase d (19, 26) and Dr. G. Wright, personal communication), was inhibitory to both enzymes from *L. mexicana*. The Pol A enzyme was more sensitive to COMDP than the pol B with IC_{50}'s of 150 and 200 1M, respectively (Table 2).

The response of these *L. mexicana* enzymes to non-specific inhibitors showed the unique properties of each enzyme (Table 2). Hemin, a critical nutritional component of the leishmanial growth media (10) was found to inhibit both enzymes, inhibiting pol B with an IC_{50} of 60 1M versus an IC_{50} of 90 1M for the pol A. Hemin inhibits DNA synthesis reversibly by binding DNA polymerase and causing it to dissociate from the template (27). Suramin, a drug used in the treatment of trypanosomiasis that has also been found to be a strong competitive inhibitor of the reverse transcriptase of a number of animal retroviruses (28), was found to be a potent inhibitor of the *L. mexicana* DNA polymerases. Suramin gave an IC_{50} of 8 1M, for pol A and 3 1M, for pol B (Table 2).

Our characterization studies have shown the *L. mexicana* pol A and pol B to differ from each other in molecular weight, pH optimum,

template specificity, and response to salt and inhibitors. In addition, our studies have shown that pol A and pol B share similar properties such as pH optimum, molecular weight, and sensitivity to specific inhibitors such as NEM with their mammalian counterparts. The assignment of pol A to a specific class among the eukaryotic DNA polymerases is made difficult by its utilization of template (Table 1) and by the particular response of this enzyme to inhibitors (Table 2). Although this high molecular weight enzyme shows a-like properties such as inhibition by NEM and salt, insensitivity to ddTTP, and preference for Mg^{+2} and activated DNA, it also displays characteristics that do not fit the a type. Pol A also shows characteristics of the d type, such as resistance to aphidicolin and utilization (although at low levels, Table 1) of ribonucleotide template when Mn^{+2} is the divalent cation. On the other hand, the low sensitivity to BuPdGTP and the somewhat high sensitivity to COMDP point toward characteristics of the DNA polymerases d and e (24).

Pol B can be more easily classified as a b-like enzyme based on its low molecular weight, resistance to NEM, and sensitivity to ddTTP. However, Pol B failed to crossreact with an anti-recombinant mouse DNA polymerase b antiserum using enzyme neutralization studies (12). Using enzyme neutralization studies, as well as immunodiffusion and immunoelectrophoresis, Chang and Bollum showed that *T. brucei* DNA polymerase b did not crossreact with an antiserum against calf thymus DNA polymerase b (29).

Observations of differences with the mammalian polymerase have been made on the enzymes of other protozoans (11-18 24,29)suggesting that DNA replication in higher eukaryotes and protozoans may differ.

Such differences are being characterized in this laboratory in the search for potential anti-parasitic agents.

Table 1.

Template specificity of the *L. mexicana* DNA polymerases A and B. Concentrations of divalent cations were 8 mM $MgCl_2$ or 0.5 mM $MgCl_2$. Adapted from Nolan et al (7) and Nolan and Rivera (8).

TEMPLATE-PRIMER	Labelled Nucleotide	Divalent Cation	% of Activity Relative to activated DNA with [*methyl*-^{3}H]dTTP	
			DNA pol A	DNA pol B
Activated DNA	dTTP	Mg^{+2}	100	100
	dTTP	Mn^{+2}	18	0
poly(rA)·oligo$(dT)_{10}$	dTTP	Mg^{+2}	3	0.9
	dTTP	Mn^{+2}	31	0.9
poly(dA)·oligo$(dT)_{12-18}$	dTTP	Mg^{+2}	99.2	149
	dTTP	Mn^{+2}	27.5	0
Activated DNA	dGTP	Mg^{+2}	1.76	17
	dGTP	Mn^{+2}	0.4	0
poly(rC)·oligo$(dG)_{10}$	dGTP	Mg^{+2}	0	2
	dGTP	Mn^{+2}	1.3	0
poly(dC)·oligo$(dG)_{12-18}$	dGTP	Mg^{+2}	57.8	650
	dGTP	Mn^{+2}	5.4	0.9

Table 2.
IC_{50} values of several DNA polymerase inhibitors on the *L. mexicana* Pol A and pol B (7,8). The IC_{50} values for the mammalian DNA polymerases indicate representative data obtained from published values in the literature (2,19,21,22,25). Enzymes found to be resistant to inhibition were marked as **R**.

	DNA polymerase						
	Mammalian					*L. mexicana*	
Inhibitor	a	b	c (1M)	d	e	A	B (1M)
AraCTP	-[a]	-[a]	-	-	-	R	>500 1M
Aphidicolin	2	R	R	2	15	R	R
NEM	<100	>10 mM	<100	<1000	-	<1000	R
BuPdGTP	.3	-[b]	>100	>10	>10	100	5.4
ddTTP	R	2	.05	R	-	R	7.5
PAA	71-710[c]	R	71-710[c]	-	-	1500	R
FPAA	20	-	-	2	-	130	R
BrPAA	160	-	-	20	-	R	R
ClPAA	120	-	-	70	-	R	R
F_2PAA	300[d]	-	-	200[d]	-	R	R
COMDP	300	-	-	40	200[e]	150	200
Suramin	-	-	-	-	-	8	3
Hemin	-	-	-	-	-	90	60

[a]It has been reported that as a rule, mammalian polymerase b is less sensitive than polymerase a with K_i values ranging from 2 - 4 1M for polymerase a versus 13 - 32 1M for polymerase b (2). [b]To our knowledge there is no published data on the inhibition of polymerase b by BuPdGTP. However, it has been reported that polymerase b is weakly inhibited by BuPdGTP (24). [c]Concentration range at which DNA polymerases a and c are reportedly inhibited by PAA (2). [d]Values obtained using poly(dA)·oligo$(dT)_{12-18}$ as the template (21). [e]The same study reported 93% inhibition of DNA polymerase d from Hela cells by 15 1M COMDP using poly(dA)·oligo(dT) (22).

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